

# Histone Deacetylase Activity and Phytotoxic Effects Following Exposure of Duckweed (*Lemna paucicostata* L.) to Apicidin and HC-Toxin

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## ABSTRACT

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The effects of two cyclic tetrapeptide fungal toxins, apicidin (from *Fusarium* spp.) and HC-toxin (from *Cochliobolus carbonum*), on duckweed (*Lemna paucicostata* L.) were examined. Both toxins inhibited histone deacetylase (HD) activity from duckweed plantlets; the effective concentration (EC<sub>50</sub>) for inhibition of HD was 5.6 and 1.1  $\mu$ M for

apicidin and HC-toxin, respectively. Approximately 65 and 85% of in vitro HD activity was inhibited by 50  $\mu$ M apicidin or HC-toxin, respectively. Exposing duckweed for 72 h to apicidin or HC-toxin (25 or 50  $\mu$ M) enhanced cellular leakage, impaired chlorophyll synthesis, and inhibited growth (cell division). At equivalent concentrations, the effects of HC-toxin were more pronounced than those of apicidin. In fronds, 72 h of exposure to 50  $\mu$ M apicidin resulted in chloroplast deterioration indicated by loss of orientation and excess starch accumulation. In roots, a 72-h treatment with 50  $\mu$ M apicidin resulted in the loss of the root cap and increased vacuolization and starch accumulation in plastids.

Apicidin (Fig. 1) is a cyclic tetrapeptide toxin recently isolated from *Fusarium sambucinum* and *F. semitectum* (22,26). *F. sambucinum* (11,20) causes dry rot of potato and fruit blotch of strawberry, whereas *F. semitectum* (9) causes pod and collar rot of soybean. Although both fungal species produce apicidin, it has not been established that this toxin plays a role in plant disease. Feeding apicidin to rats causes hemorrhagic symptoms (22). Protozoan parasites of the subphylum Apicomplexa, including *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Eimeria tenella*, are very sensitive to apicidin (7). It has been proposed that the anti-protozoan activity of apicidin is due to its ability to inhibit histone deacetylase (HD) (7). By regulating the level of acetylation of core histones associated with DNA, HD is thought to play a key role in regulating nuclear transcription of genes. The results of Darkin-Rattray et al. (7) suggest that apicidin prevents in vitro development of intracellular apicomplexan parasites by causing hyperacetylation of core histones.

HC-toxin (Fig. 1), a cyclic tetrapeptide peptide isolated from *Cochliobolus carbonum* (formerly *Helminthosporium carbonum*) race 1, causes leaf spot in maize that is homozygous recessive at the *Hm* locus (23). In vitro assays indicate that HC-toxin is a potent inhibitor [effective concentration (EC<sub>50</sub>)  $\approx$  5  $\mu$ M] of maize HD (5). It has been proposed that by blocking HD and thereby interfering with the regulation of gene expression, HC-toxin suppresses the ability of susceptible maize genotypes to mount a defense response to the invading fungus (5,23). Other research has shown that HC-toxin inhibits HD from apicomplexan parasites (7), mammalian cells (5), and the fungus *Physarum polycephalum* (5,18).

The objectives of this research were to investigate the in vitro effects of apicidin and HC-toxin on duckweed HD, examine phytotoxic effects of both toxins on duckweed at the whole plant level, and characterize the effects of apicidin at the ultrastructural level. Duckweed (*Lemna paucicostata* L.) was chosen as a bio-assay species because previous research demonstrated its utility as a model plant for investigating the effects of fungal toxins at both the whole plant and ultrastructural level (2,3,21,27). A preliminary report of the effects of apicidin on duckweed were presented earlier (1).

## MATERIALS AND METHODS

**Chemicals.** The synthetic peptide fragment, Ac-Gly-Ala-Lys-Arg-His-Arg-Lys-Val-NH<sub>2</sub>, used as a substrate for measuring HD activity, was synthesized and purified by the Microchemical Facility, Institute of Human Genetics, University of Minnesota. [<sup>3</sup>H]-acetic anhydride (specific activity 64 mCi/mmol), used for labeling the synthetic peptide, was purchased from ICN Biomedicals (Costa Mesa, CA). Prep Sep C<sub>18</sub> columns were purchased from Fisher Scientific (Hanover Park, IL). HC-toxin and Sephadex G-25 were purchased from Sigma Chemical (St. Louis). Apicidin was isolated and purified from *Fusarium* spp. as described in detail by Park et al. (22). Apicidin was also provided by Merck Research Laboratories, Rahway, NJ.

**Labeling of HD substrate.** The synthetic peptide fragment (residues 14 to 21) of histone H4 (Ac-Gly-Ala-Lys-Arg-His-Arg-Lys-Val-NH<sub>2</sub>) was chemically acetylated as described by Sendra et al. (25) with modifications. Purified peptide (10 mg) was dissolved in 0.2 ml of 20 mM triethanolamine (pH 8.2) and added to 78  $\mu$ l of [<sup>3</sup>H]-acetic anhydride. The reaction was incubated for 30 min at 23°C, and 50  $\mu$ l of 1.25 M HCl and 2.0 ml of ice cold acetone were added to precipitate the peptide. The solution was centrifuged (2,000  $\times$  g, 10 min) at 4°C. The supernatant was dis-

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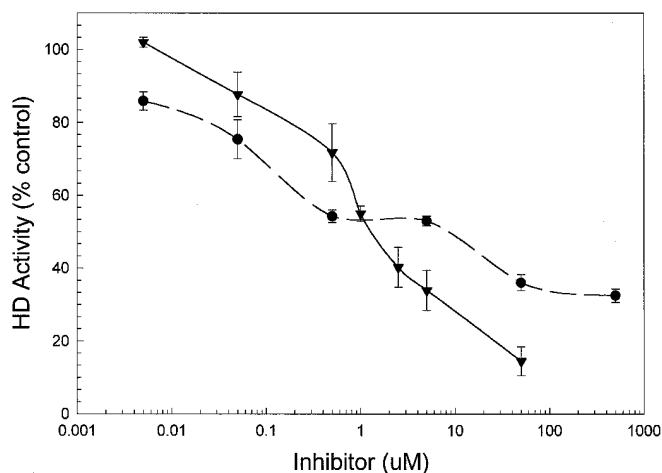
carded, and the pellet was washed twice with 1.0 ml of acetone. The labeled peptide was resuspended in buffer A (15 mM Tris-HCl, pH 7.8, 10 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol). To remove contaminating [ $^3\text{H}$ ]-acetic acid, 0.3-ml aliquots were applied to Prep Sep  $\text{C}_{18}$  columns equilibrated in  $\text{H}_2\text{O}/0.1\%$  trifluoroacetic acid (TFA). Columns were washed with 2.0 ml of  $\text{H}_2\text{O}/0.1\%$  TFA, and the peptide was eluted with 2.0 ml of 70% acetonitrile/0.1% TFA. The solvent was removed by evaporation under a stream of nitrogen, and the labeled peptide was dissolved in buffer A. The specific activity of the acetylated histone H4 peptide was  $6.6 \mu\text{Ci}/\mu\text{mol}$ .

**Bioassays.** Duckweed bioassays were conducted as described by Tanaka et al. (27,28). Ten duckweed plantlets were transferred to small petri dishes (3.5 cm ID) containing 3 ml of the following: half-strength Hutners medium (12) alone, medium containing 0.05% dimethyl sulfoxide (DMSO), or medium containing 0.05% DMSO plus 25 or 50  $\mu\text{M}$  apicidin or HC-toxin. Petri dishes were covered with three layers of cheesecloth and incubated at  $25^\circ\text{C}$  under continuous light ( $115 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in a growth chamber. Electrolyte leakage was determined at 12-h intervals with a conductivity meter. To determine chlorophyll, duckweed plantlets (colonies) were removed from the bathing medium, blotted with paper towels, and extracted by incubating overnight in 5 ml of DMSO. Total chlorophyll was determined as described by Hiscox and Israelstam (10) and expressed on a gram fresh weight basis. Growth inhibition was measured by determining duckweed fresh weights at the beginning and end of the experiment. Duckweed bioassay experiments were repeated with three replicates per treatment. Each replicate contained 10 duckweed colonies with each colony containing three fronds. The results are reported as the mean  $\pm\text{SE}$  of two experiments.

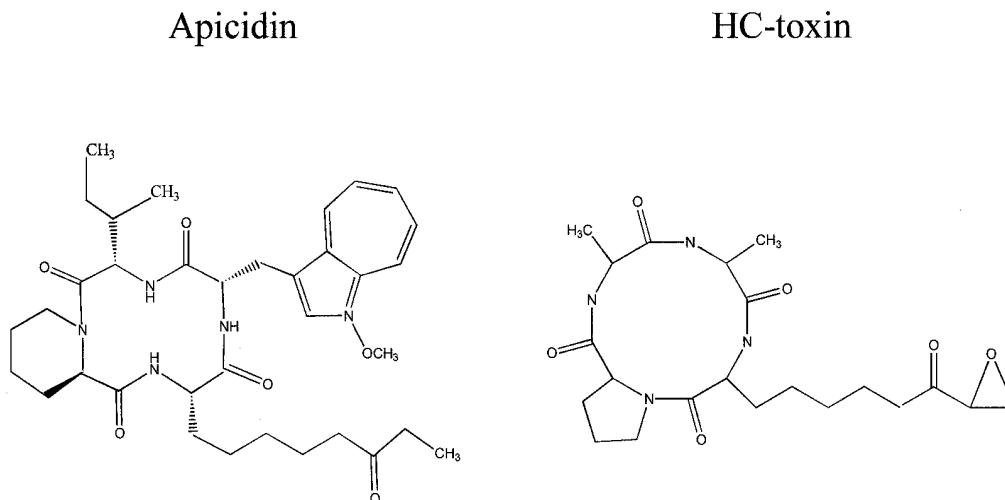
**Ultrastructural effects.** Duckweed was grown as described previously in half-strength Hutners medium containing 0.05% DMSO or 0.05% DMSO plus 50  $\mu\text{M}$  apicidin. Tissue was sampled at 12-h intervals over a 72-h period. Tissue was placed in a drop of 0.4% glutaraldehyde fixative in 0.01 M cacodylate acid buffer, pH 7.0, and cut in the fixative with a single edge razor blade. Tissue segments were transferred to a vial containing fresh buffered fixative. Tissue segments were fixed for 1.5 h and rinsed six times in glutaraldehyde-free buffer throughout a 1-h period. Tissue segments were then postfixed for 1 h in 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.0. After rinsing in distilled water (six changes), within a 1-h period, the tissue was dehydrated in a graded acetone series. Once in 100% acetone, the tissue was embedded and polymerized in Spurr's medium. Semi-thin sections were obtained with a diamond knife in an ultramicro-

tome (Leica Ultracut E; Leica Microsystems, Inc., Bannockburn, IL) and mounted on glass slides, stained with toluidine blue, and observed with a light microscope. Thin sections were stained with uranyl acetate and Reynold's lead citrate and observed and photographed in a transmission electron microscope (Zeiss EM10 CR; LEO Electron Microscopy, Inc., Thornwood, NJ).

**Extraction of HD.** Duckweed was grown at  $23^\circ\text{C}$  under constant light (fluorescent, PPFD =  $110 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in glass baking trays ( $34 \times 23 \text{ cm}$ ) with sterile half-strength Hutners medium, pH 6.0 (12). Every 3 to 4 days, several duckweed fronds were transferred to fresh medium and the remaining plants were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until extracted for HD activity. HD was extracted from a total cellular homogenate of duckweed plantlets according to the method of Kolle et al. (15). All steps were performed at  $4^\circ\text{C}$ . Briefly, frozen duckweed tissue (10 g) was ground in liquid nitrogen with a mortar and pestle. The ground tissue was added to 40 ml of buffer B (15 mM Tris-HCl, pH 7.8, 10 mM NaCl, 0.25 mM EDTA, and 10 mM 2-mercaptoethanol). The solution was brought to 0.7 M NaCl by adding 8.5 ml of 4 M NaCl in buffer B. The extract was stirred for 60 min, and filtered sequentially through two layers each of cheesecloth and miracloth. The filtrate was centrifuged ( $20,000 \times g$ , 20 min). Aliquots (2.0 ml) of the resulting supernatant were desalted on



**Fig. 2.** Effect of apicidin and HC-toxin on histone deacetylase (HD) activity measured in duckweed extracts. Effective concentration ( $\text{EC}_{50}$ ) values for apicidin (●) and HC-toxin (▼) were 5.6 and 1.1  $\mu\text{M}$ , respectively. Values represent the mean  $\pm\text{SE}$  of three separate isolations.



**Fig. 1.** Chemical structure of apicidin and HC-toxin.

10-ml Sephadex G-25 columns equilibrated in buffer A. The desalted crude extract was used in the HD assays.

**HD assays.** The assay mixture (100  $\mu$ l) contained 15 mM Tris-HCl (pH 7.8), 10 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 1% DMSO, 0.06 mM Ac-Gly-Ala-Lys( $\epsilon$ - $^3$ H)Ac)-Arg-His-Arg-Lys( $\epsilon$ - $^3$ H)Ac)-Val-NH<sub>2</sub> specific activity 6.6  $\mu$ Ci/ $\mu$ mol), and duckweed extract with or without inhibitors (apicidin or HC-toxin) dissolved in 100% DMSO. The reaction was incubated for 1 h at 22°C, and stopped by the addition of 20  $\mu$ l of 1.0 M HCl/0.4 M acetic acid. The reaction was linear during this period. Released [ $^3$ H]-acetate was extracted with 0.8 ml of ethyl acetate. After centrifugation (10,000  $\times$  g, 5 min), 600  $\mu$ l of the organic phase was counted in 5 ml of Ecolume. Uninhibited HD-catalyzed acetate release (approximately 650 dpm/reaction) was determined by subtracting the radioactivity measured in zero extract controls.

## RESULTS

**Effects on HD.** A high ionic strength buffer described by Kolle et al. (15) was used for extracting HD from duckweed. Previous research demonstrated the requirement for high ionic strength for effective extraction of active HD from a variety of plant tissue sources including maize embryos (4,15). However, because high ionic strength inhibits HD activity (15), it was necessary to desalt the crude duckweed fraction prior to assay. An [ $^3$ H]-acetylated H4 peptide (residues 14 to 21) was used as a substrate for in vitro assays. This substrate was previously used to assay for HD activity from calf thymus (13) and *E. tenella* (7). Using this assay, EC<sub>50</sub> values of 1.1 and 5.6  $\mu$ M were determined for HC-toxin and apicidin, respectively (Fig. 2). The pattern of inhibition of in vitro activity differed between the two cyclic tetrapeptides. Apicidin was more effective than HC-toxin at lower concentrations and less effective at high concentrations. At 50  $\mu$ M, apicidin and HC-toxin inhibited HD activity by 65 and 85%, respectively. Approximately 30% of HD activity was not inhibited by 500  $\mu$ M apicidin.

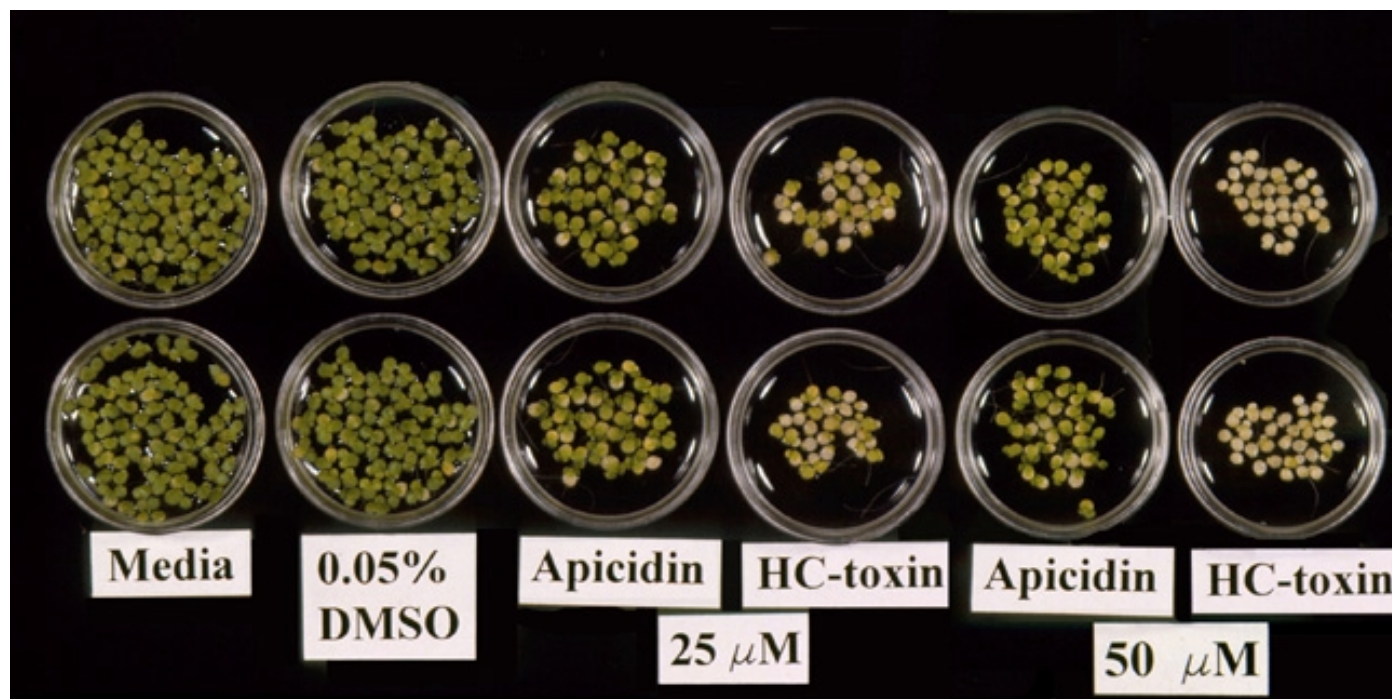
**Effects on duckweed.** Visual observation indicated that both toxins inhibited growth and chlorophyll synthesis after 72 h of exposure (Fig. 3). Therefore, effects of 72 h of exposure to api-

cidin (25 or 50  $\mu$ M) or HC-toxin (25 or 50  $\mu$ M) on growth, chlorophyll synthesis and membrane permeability (cellular leakage) were examined. At equivalent concentrations, the effects of HC-toxin were greater than apicidin. After 72 h of exposure to 25 or 50  $\mu$ M apicidin, growth (cell division) was inhibited by 50 and 58%, respectively (Fig. 4A). For HC-toxin, growth inhibition was 71 and 78%, respectively. Chlorophyll concentration in fronds was reduced by 10 and 53% after 72 h of exposure to 25 and 50  $\mu$ M apicidin, respectively (Fig. 4B). Again, the effect of equivalent concentrations of HC-toxin was greater than that observed for apicidin with a 72-h exposure to 25 or 50  $\mu$ M HC-toxin, reducing chlorophyll content by 82 and 93%, respectively. At concentrations of 25 and 50  $\mu$ M, enhanced cellular leakage of electrolytes was apparent after 24 h of exposure to both toxins (Fig. 5). Electrolyte leakage from HC-toxin-treated plantlets was greater than from apicidin-treated plantlets.

**Effects of apicidin on ultrastructure.** A 72 h time course of the effects of exposure to 50  $\mu$ M apicidin on duckweed frond and root ultrastructure was conducted (Figs. 6 and 7). In frond tissue, 36 h was the earliest time at which there was a consistent difference between the control (Fig. 6A) and the treated tissue (Fig. 6B). Many organelles in apicidin-treated tissues appear less distinct. The chloroplasts of apicidin-treated tissues contain starch grains that are larger than those of the control tissues.

The treated tissue appeared much the same after 48 h as it did at 36 h (Fig. 6C). At 60 h, control (Fig. 6D) tissue had dense cytoplasm with normal appearing organelles. Treated tissue (Fig. 6E and F) had less dense cytoplasm, and vacuoles lacked a tonoplast; however, some recognizable organelles were present. At 72 h, controls (Fig. 6G) and treated (Fig. 6H) tissue were very different. Nothing in the treated tissue other than chloroplast membrane remnants, starch grains, and cell wall material was recognizable.

Because duckweed roots were immersed in the treatment solution, they were more directly exposed to apicidin during incubation than were fronds. As a result, effects on root tip ultrastructure were seen earlier during the time course. After 12 h, control root tip cells (Fig. 7A) contain dense cytoplasm with many small vacuoles. Their plastids have well-stained thylakoids, dense stroma, and small starch grains. A light micrograph (Fig. 7A, insert)



**Fig. 3.** Effect of 25 or 50  $\mu$ M apicidin or HC-toxin on duckweed growth and chlorophyll synthesis. Visual comparison of the effect of 72 h of exposure to Hutner's medium alone, medium plus 0.05% dimethyl sulfoxide (DMSO), or medium containing 0.05% DMSO plus 25 or 50  $\mu$ M apicidin and HC-toxin.

shows the contrast between the dense cells of the root tip and the highly vacuolated cells of the root cap. At 12 h, apicidin-treated root tip tissue (Fig. 7B) appears much the same as the untreated controls, except for a slight increase in the number of small vacuoles, although this varies between cells. Treated root tip tissue incubated for 36 h (Fig. 7C) shows some increase in vacuole size and this effect is observable at the light level. A micrograph of 60-h treated material (Fig. 7D) shows that some root cap material survives with only slight disruptions in ultrastructure (enlarged starch grains and vacuoles). At 72 h (Fig. 7E), electron micrographs of treated root tip tissue show that displacement of the cytoplasm by enlarged vacuoles has resulted in a spongy appearance. Nuclei and numerous starch containing plastids are discernable. A light micrograph depicts the usual appearance of root tips during longer stages of incubation in the toxin; the root cap is missing, and the root tip itself appears highly vacuolated compared with the control (Fig. 7A, insert). Control root tip tissue harvested at 72 h (Fig. 7F) is indistinguishable from earlier controls; it has dense cytoplasm with numerous small vacuoles, and all organelles are intact. Their plastids contain well-organized thylakoids and dense stroma.

## DISCUSSION

The results of this study provide, to our knowledge, the first report that the cyclic tetrapeptide apicidin inhibits HD activity in higher plants. The  $EC_{50}$  value for inhibition of duckweed HD activity was 5.6  $\mu$ M. Compared with apicidin, HC-toxin was a more potent inhibitor of duckweed HD with an  $EC_{50}$  value of

1.1  $\mu$ M. An  $EC_{50}$  value of approximately 5  $\mu$ M was reported for HC-toxin inhibition of HD activity from a partially purified fraction from maize seedlings (5). Apicomplexan HD activity is more sensitive to both toxins than higher plant HD.  $EC_{50}$  values for inhibition of HD from extracts of *E. tenella* were 0.7 and 30 nM for apicidin and HC-toxin, respectively (7). In contrast to duckweed HD, apicomplexan HD is more sensitive to apicidin than HC-toxin.

In addition to the difference in effectiveness of HC-toxin and apicidin as inhibitors of duckweed HD, the pattern of inhibition also differed. For HC-toxin, inhibition was approximately sigmoidal; however, for apicidin, a biphasic pattern of inhibition was apparent. Apicidin was more effective at inhibiting HD activity at low concentrations, but less effective at high concentrations. Even at 500  $\mu$ M apicidin, approximately 30% of HD activity was not inhibited.

Maize (4,5,16), pea (25), and yeast (6) have multiple forms of HD. In maize, all forms are equally sensitive to HC-toxin (5). Assuming multiple forms of HD are present in duckweed, our results suggest that the isoforms do not have equal sensitivity to both toxins. The results suggest that a component (one or more isozymes) of HD activity is not inhibited by apicidin. In yeast, HD isoforms A and B differ in sensitivity to the HD inhibitor trichostatin A (6). Further research with purified higher plant HD isoforms will be required to determine whether apicidin is a selective inhibitor of one or more isoforms.

The effect of cyclic tetrapeptides on duckweed HD was evaluated in vitro by measuring the deacetylation of a highly conserved region (residues 14 to 21) of the H4 histone. The substrate peptide was synthesized and acetylated with [ $^3$ H]-acetate. This acetylated octapeptide was previously used as a substrate for evaluating the effect of apicidin on apicomplexan HD (7). Our results suggest that the acetylated H4 histone peptide is a suitable substrate for higher plant HD. Most studies to evaluate the effects of various inhibitors on higher plant HD used a [ $^3$ H]-acetate-labeled total histone fraction from chicken erythrocytes (5,25). However, obtaining this substrate requires considerably more effort than in vitro synthesis and acetylation of the H4 histone peptide. The  $EC_{50}$  value that we obtained for inhibition of duckweed HD using the acetylated H4 histone peptide (1.1  $\mu$ M) is similar to that obtained for inhibition of maize HD ( $\approx$ 5  $\mu$ M) using the acetylated chicken erythrocyte histone fraction (5).

Phytotoxic effects of HC-toxin and apicidin were observed at the whole plant level when plantlets were exposed for 72 h to

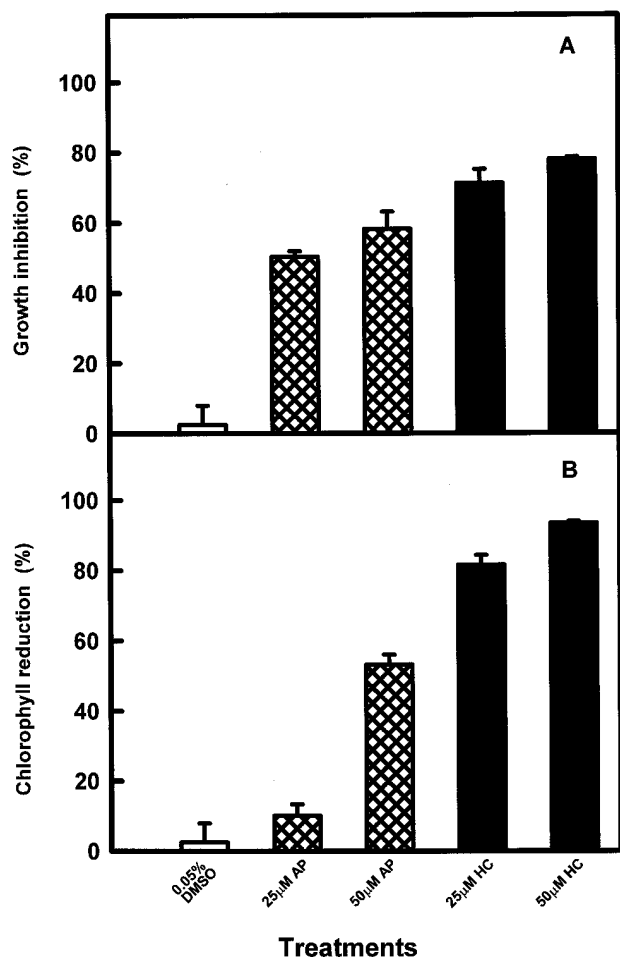


Fig. 4. Effects of 72-h treatment with apicidin (AP) and HC-toxin (HC) on A, growth and B, chlorophyll content of duckweed. Growth inhibition and percent chlorophyll were determined on a gram fresh weight basis.

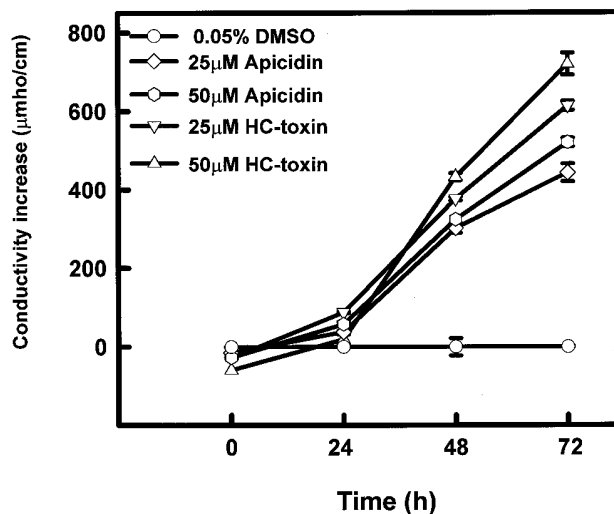
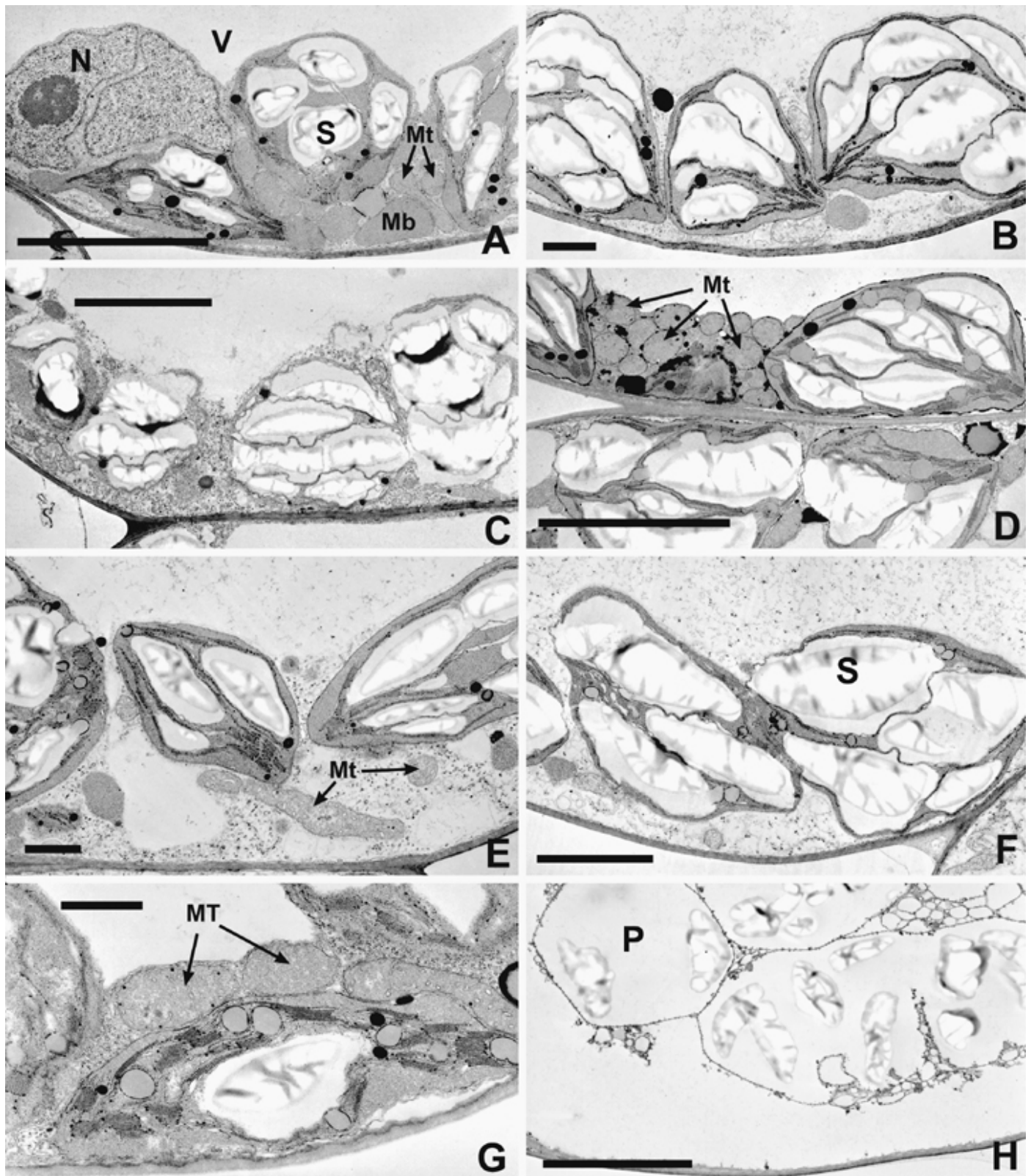


Fig. 5. Time course of effect of apicidin (25 or 50  $\mu$ M) and HC-toxin (25 or 50  $\mu$ M) on cellular leakage of duckweed plantlets as measured by the conductivity increase of the external medium.

25 or 50  $\mu\text{M}$ . Based on in vitro assays, 50  $\mu\text{M}$  apicidin or HC-toxin inhibited HD activity by 65 and 85%, respectively. It is very likely that internal concentrations of apicidin and HC-toxin were less than external concentrations in the medium due to restrictions

in movement of the two cyclic tetrapeptides across membranes. Inhibition of HD by apicidin or HC-toxin would have resulted in hyperacetylation of core histone proteins of duckweed nuclear DNA, which in turn would interfere with gene expression. The



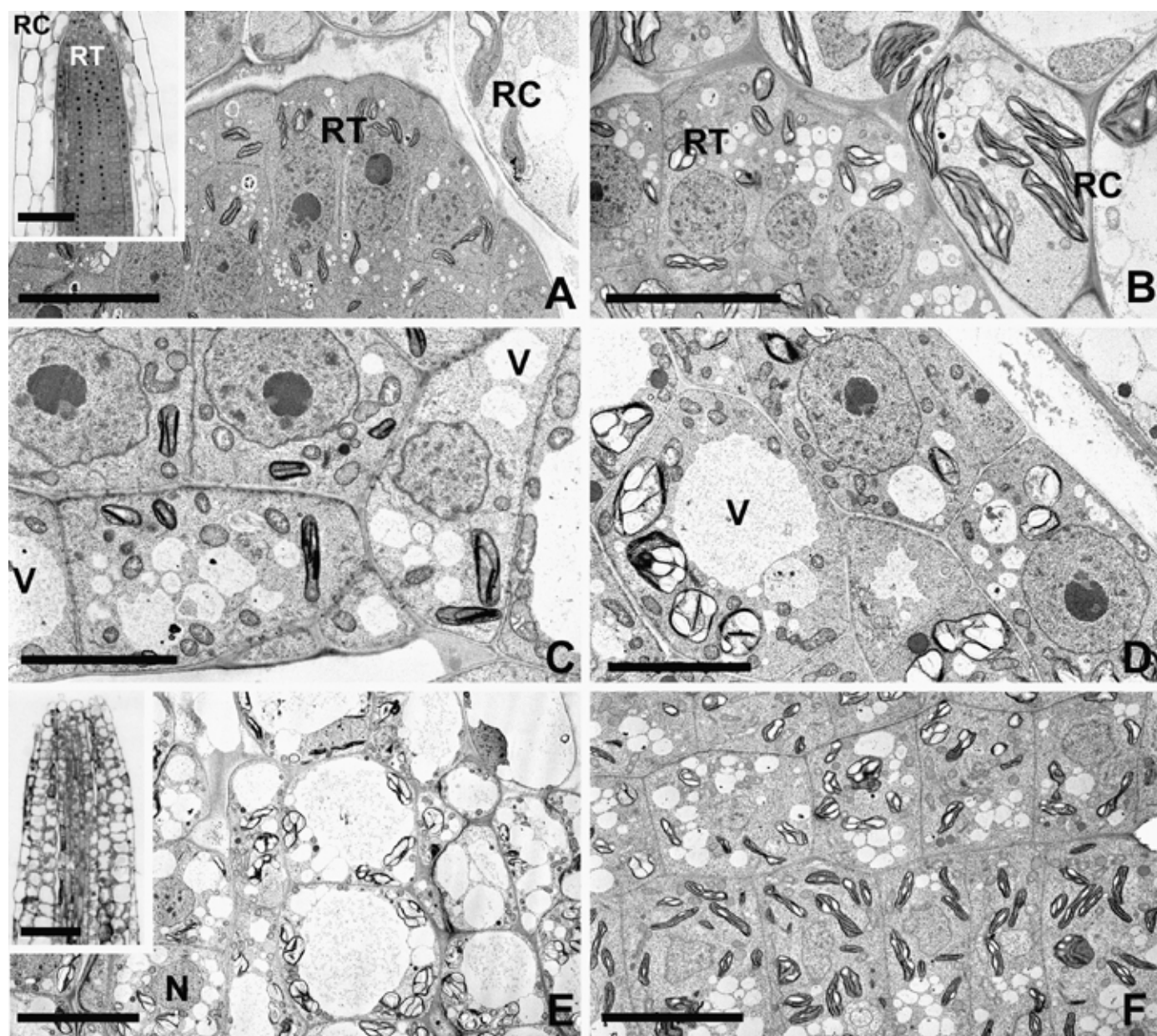
**Fig. 6.** Ultrastructural effects of 50  $\mu\text{M}$  apicidin on duckweed fronds. **A**, Transmission electron microscopy (TEM) of 36-h control. Plastids, nuclei, mitochondria, and other cytoplasmic organelles are present and in good condition. Bar = 5  $\mu\text{m}$ . **B**, TEM of 36-h treated tissue. Plastids appear normal but the cytoplasm is less dense. Bar = 1  $\mu\text{m}$ . **C**, TEM of 46-h treated tissue. Plastids and cytoplasm appear more damaged. Bar = 5  $\mu\text{m}$ . **D**, TEM of 60-h control. Bar = 5  $\mu\text{m}$ . **E**, TEM of 60-h treated tissue. Although cell damage was variable, cytoplasmic integrity was adversely affected. Bar = 1  $\mu\text{m}$ . **F**, TEM of 60-h treated tissue. Here, reasonably intact chloroplasts exist in remnants of destroyed cytoplasm. Bar = 2.5  $\mu\text{m}$ . **G**, TEM of 72-h control. Bar = 1  $\mu\text{m}$ . **H**, TEM of 72-h treated tissue. Although the degree of damage varied among cells, lack of cytoplasm, starch grains, and remnants of chloroplasts was commonly observed. Bar = 5  $\mu\text{m}$ .

fact that at both the enzyme and plant level, greater toxicity was observed for HC-toxin than apicidin is consistent with HD being the site of action of both toxins.

At 50  $\mu\text{M}$ , apicidin or HC-toxin inhibited *in vitro* HD activity by 65 and 85%, respectively. These toxins also elicited similar levels of inhibition of duckweed growth. After 72 h of exposure to 50  $\mu\text{M}$  apicidin or HC-toxin, growth had been inhibited by 58 and 78%, respectively. Most likely, reductions in plantlet biomass reflect inhibitory effects of both toxins on cell division and daughter cell formation.

Other research has indicated that various HD inhibitors block growth and cell division of both plant and mammalian cells. In maize genotypes susceptible to HC-toxin, root growth (elongation) was inhibited 50% by approximately 2  $\mu\text{M}$  HC-toxin (17,30).

In a resistant maize genotype (homozygous dominant at the *Hm* locus),  $\text{EC}_{50}$  values were 115  $\mu\text{M}$  due to toxin detoxification in planta via carbonyl reductase (17). Apicidin (50 to 100 nM) inhibited mammalian (HeLa) cell proliferation *in vitro* by 50% (7), and HC-toxin (10 nM) caused half-maximal inhibition of cell division of mouse mastocytoma cells (30). Trichostatin A, a *Streptomyces* metabolite that blocks mammalian HD, inhibits the cell cycle of rat fibroblasts in the G1 and G2 phases (31,32). Trichostatin A also causes accumulation of acetylated histones species in a variety of mammalian cell lines. Trapoxin, a cyclic tetrapeptide fungal metabolite that inhibits mouse HD, caused accumulation of highly acetylated core histones in a variety of mammalian cell lines, and arrested the cell cycle of rat fibroblasts in both the G1 and G2 phases (14).



**Fig. 7.** Light and electron micrographs of control and apicidin-treated duckweed root tips. **A**, Control root tip (12 h). This tissue is composed of healthy cells in which cytoplasm is dense and contains numerous small vacuoles. The plastids have well stained thylakoids, dense stroma, and a few have small starch grains. Root tip (RT) tissue is very distinct from root cap (RC) tissue in that the latter is highly vacuolated. Bar = 10  $\mu\text{m}$ . Inset: light micrograph of 12-h control root tip showing root cap. Bar = 50  $\mu\text{m}$ . **B**, Apicidin-treated root tip (12 h). Except for the slight enlargement of vacuoles, cytosol and organelles appear normal. Bar = 10  $\mu\text{m}$ . **C**, Apicidin-treated root tip (36 h). Vacuoles (V) have become even more enlarged. Bar = 5  $\mu\text{m}$ . **D**, Treated root tip (60 h). Cells have enlarged vacuoles and more starch accumulation in plastids. Bar = 5  $\mu\text{m}$ . **E**, Apicidin-treated root tip (72 h). Transmission electron microscopy reveals a spongy appearance to the root, due to displacement of the cytoplasm by vacuoles. Nuclei (N) and numerous starch-containing plastids are present. Bar = 10  $\mu\text{m}$ . Inset: Light micrograph of 72-h treated root tip in cross section. The root cap is missing and the tissue appears spongy. Bar = 50  $\mu\text{m}$ . **F**, Control root tip (72 h). Except for an increase in the size of starch grains in plastids, tissue is very similar to the 12-h control. Bar = 5  $\mu\text{m}$ .

Another major effect of treatment with apicidin and HC-toxin was a reduction in chlorophyll content of duckweed fronds. HC-toxin was more effective at blocking chlorophyll synthesis than apicidin. A 72-h exposure to 50  $\mu$ M apicidin or HC-toxin reduced chlorophyll content of fronds by 53 and 93%, respectively. Previous research demonstrated that HC-toxin (20  $\mu$ g/ml) blocked chlorophyll synthesis in etiolated leaves of a maize genotype susceptible to *Cochliobolus carbonum* (24). It specifically blocked the synthesis of  $\delta$ -aminolevulinic acid, the light inducible and first committed intermediate in chlorophyll synthesis.

To our knowledge, this is the first study to evaluate the effects of an HD inhibitor on the ultrastructure of higher plants. One of the first observable effects of apicidin at the ultrastructural level was its deleterious effects on membranes. Evidence of this was the disappearance of the tonoplast in apicidin-treated fronds and roots. The enhanced electrolyte leakage that occurred in response to apicidin treatment suggests that the semipermeability properties of the plasma membrane were impaired. Another effect of apicidin was expressed at the plastid level. In fronds and roots, apicidin treatment resulted in starch accumulation in chloroplasts and plastids, respectively. The starch inclusions continued to enlarge in treated tissue until the later stages of the 72-h time course. This suggests that disruption of photosynthesis is not an early manifestation of the toxin.

Inhibiting duckweed HD with apicidin and HC-toxin suppressed growth, blocked chlorophyll synthesis, and adversely affected ultrastructure. Because HD is thought to play a key role in regulating gene expression, it should not be surprising that HD inhibitors would have diverse and wide ranging effects at the cellular level. Although the results of this study are consistent with the effects of both toxins being due to inhibition of duckweed HD, experiments need to be conducted to determine whether these toxins cause hyperacetylation of nuclear DNA core histones *in vivo*.

Although there is substantial evidence that HD plays a key role in establishing the acetylation/deacetylation equilibrium of core histones, just how this dynamic equilibrium regulates chromatin structure and DNA transcription is not understood. It is proposed that acetylation of core histones regulates accessibility of chromatin regions to regulatory proteins like transcription factors (8,19). Others have proposed that histone acetylation plays a larger role in the expression of inducible genes than it does with constitutive housekeeping genes (5). Apicidin and HC-toxin may become useful tools to advance understanding of the role of core histone acetylation in regulating DNA transcription and ultimately gene expression.

It is well established that HC-toxin is a critical determinate of the virulence of *Cochliobolus carbonum* race 1 to maize with the *hm/hm* genotype (29). Earlier research demonstrated that HC-toxin inhibits maize HD both *in vitro* (5) and *in vivo* (23). Our results provide further evidence that HD is the target site of HC-toxin. *In vitro* assays indicated that HC-toxin was a potent inhibitor of duckweed HD. By regulating the acetylation/deacetylation equilibrium of core (nucleosomal) histones, HD is considered to play an important role in regulating gene transcription (16,19). The mechanism whereby inhibition of HD acts to enhance disease susceptibility of maize with the *hm/hm* genotype is not known. It has been proposed that inhibition of HD by HC-toxin interferes with the expression of genes needed to mount a defense response to *Cochliobolus carbonum* race 1 (5,23). Apicidin was recently isolated from *F. sambucinum* (22) and *F. semitectum* (26). Although *F. sambucinum* and *F. semitectum* cause disease in potato, strawberry (11,20), and soybean (9), respectively, it has not been established that apicidin plays a role in pathogenicity. Our research showed that apicidin is a potent inhibitor of plant HD. Future research will need to determine whether apicidin is a virulence factor in the diseases caused by these *Fusarium* spp. and, if so, whether it acts by interfering with plant defense responses as proposed for HC-toxin.

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